FUNCTIONAL SUBUNITS OF LIVER MICROSOMAL CYTOCHROME *P*-450: MOLECULAR WEIGHT ESTIMATE BY RADIATION INACTIVATION

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1. Introduction

Although attempts to perform elaborate studies on purification and physical properties of cytochrome *P*-450 (a mono-oxygenase related to various hydroxylation reactions) have been hampered by the difficulties in solubilizing it in a stable form, there is growing evidence that the minimum mol. wt of the cytochrome is 50 000–59 000 in the case of rat liver microsomal *P*-450 [1, 2]. Recently, one of cytochromes *P*-450 has been isolated from bovine adrenocortical mitochondria as an oligomeric protein composed of 16 subunits, each of mol.wt 53 000 [3]. In the present study, the radiation inactivation method [4, 5] was employed for estimating the molecular size of the mouse liver microsomal *P*-450. The molecular weight value obtained by this method was in good agreement with the above-mentioned values. These results are indicative of the fact that both the microsomal and mitochondrial cytochromes *P*-450 have similar subunit structure.

2. Materials and methods

2.1. *Liver microsomes*

Male mice (6–7 weeks-old, 25–30 g) of the ddY strain were injected intraperitoneally with sodium phenobarbital (3 mg/animal/day) for 4 successive days and killed 17 hr after the last injection. Pooled hepatic tissues (15 g) were homogenized with 30 ml of ice-cold 0.25 M sucrose–0.02 M phosphate buffer (pH 7.4). Microsomes obtained by a conventional differential-centrifugation method were suspended in 10 ml of the sucrose–phosphate solution and lyophilized. The dried samples were powdered in a mortar and stored at 5°C in a disiccator over $CaCl_2$. The protein content was 27% w/w.

2.2. *Determination of P-450*

The microsomal powder was suspended in 0.01 M phosphate buffer (pH 7.0) with use of a Teflon-glass homogenizer and cytochrome *P*-450 was determined by the method of Omura and Sato [6]. Linear correlation was observed between the concentration of microsomes and the magnitude of the 450 nm peak, when the suspensions of 0.3–3.0 mg protein/ml were used. The *P*-450 content of the microsomes was 2.0 nmoles/mg protein, when calculated on the basis of an extinction coefficient of 91 mM^{-1} cm^{-1}. It decreased approximately by 10% in 36 hr, when the dry powder was left standing in vacuum at room temperature. The radiation-induced decomposition of *P*-450 was corrected for this spontaneous loss of CO-binding activity.

2.3. *Irradiation*

Dry powders (each 10 mg) of the microsomes and the standard enzyme proteins were packed separately in small glass tubes, which were then evacuated at 5×10^{-5} mm Hg for 1 hr and subsequently sealed under the same vacuum. The tubes (6 × 30 mm) with the samples tightly packed on the bottom were irradiated at room tenperature with [^{60}Co] gamma-rays (5900–6000 rad/min, monitored by a conventional ferrous sulfate method). Immediately after irradiation (2–30 Mrad), the tubes were opened in air and the enzyme activity was determined. Values of D_0 (the dose required to give a residual activity of e^{-1} (36.8%) of the non-irradiated control) were obtained by drawing the best-fitting curve in semi-logarithmic plots of surviving enzyme activity versus radiation dose by the method of least squares.

2.4. *Standard enzyme proteins*

The following enzymes were purchased and used without further purification: Yeast alcohol dehydrogenase, Worthington; α-amylase of *Bacillus subtilis*, Seikagaku Kogyo; bovine liver catalase, Sigma; bovine pancreas trypsin, Sigma; rabbit muscle creatine kinase, Boehringer; bovine liver β-glucuronidase, Worthington; bovine pancreas α-chymotrypsin, Sigma; horseradish peroxidase, Sigma. The activity of these enzymes was assayed by conventional methods available for each.

3. Results and discussion

When the microsomes were irradiated with the gamma-rays, there occurred a decrease in the height of the 450 nm peak of the CO-difference spectra of the cytochrome. It is noted that inactivation of *P*-450 did not result in formation of *P*-420 (solubilized form of *P*-450) as shown in fig. 1. The color developed by carbon monoxide was stable for a few hr, even when heavily irradiated microsomes were used. With the increase in radiation dose, CO-binding activity of *P*-450 decreased exponentially, and semi-logarithmic plots of residual activity against radiation dose gave a straight line (fig. 2). The D_0 for *P*-450 was 10 Mrad and it lead to an estimate of mol. wt 57 000 for the cytochrome on the basis of log molecular weight versus log D_0 plots obtained by irradiation of standard enzyme proteins under the same conditions of experiment (fig. 3). The straight line drawn through the experimental points in fig. 3 is nearly consistent with the line drawn theoretically on the assumption of the single-hit target theory that 60 eV is required for one inactivating event. This value is comparable with that (66 eV) calculated by Kepner and Macey [5].

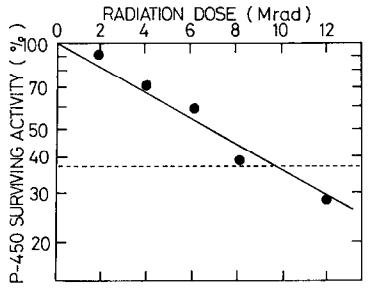

Fig. 2. Radiation inactivation of CO-binding activity of microsomal cytochrome *P*-450 as a function of radiation dose. Each point represents the average of 3 determinations.

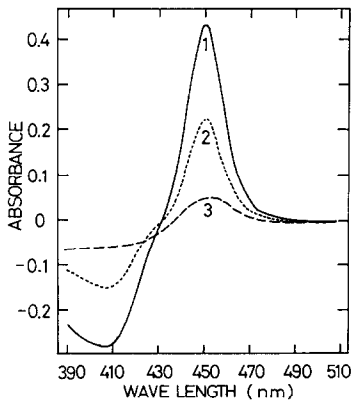

Fig. 1. Carbon monoxide difference spectra of cytochrome *P*-450 of mouse liver microsomes: 1) control non-irradiated microsomes (2.4 mg protein/ml); 2) microsomes irradiated with 4.0 Mrad gamma-rays (1.7 mg protein/ml); 3) microsomes irradiated with 12 Mrad gamma-rays (1.2 mg protein/ml).

The D_0 value for an oligomeric enzyme normally represents the minimum molecular weight of its active subunit as it was demonstrated by Blum and Alper [7]. However, under certain conditions, it changes stepwise corresponding to the polymeric structure of the enzyme as observed in case of catalase [8] and thrombin [9]. On the other hand, the presence of impurity [9] and sucrose [5] does not influence the radiosensitivity of the enzyme. Thus, the molecular

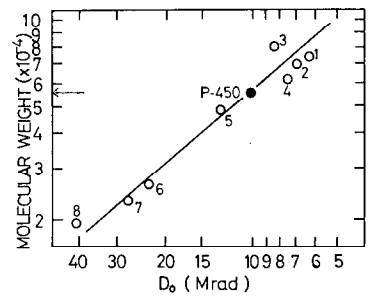

Fig. 3. Correlation between molecular weight and D_0 for various enzymes irradiated in the dry state: 1) alcohol dehydrogenase (75 000); 2) β-glucoronidase (70 000); 3) creatine kinase (81 000); 4) catalase (61 000); 5) α-amylase (49 000); 6) α-chymotrypsin (27 000); 7) trypsin (23 800); 8) peroxidase (20 000). ($\times 10^{-4}$) Represents the molecular weight of the enzyme or its subunit.

weight of malate dehydrogenase of membrane fragments of *Micrococcus lysodeikticus* determined by the radiation inactivation method was in good agreement with the molecular weight determined after the enzyme was isolated from other components of the membrane and purified in crystalline form [10].

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